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PEPTIDES FOR ACTIVATION AND INHIBITION OF δPKC

This application claims the benefit of U.S. Provisional Application No. 60/262,060 filed January 18, 2001, incorporated herein by reference in its entirety.

This work was supported in part by The National Institutes of Health Grant HL 52141. Accordingly the United States government may have certain rights in this invention.

Field of the Invention

The present invention relates to peptides effective to activate or inhibit translocation and/or function of δPKC. The present invention also relates to therapeutic compositions and methods for treating diseases or conditions which are benefited by inhibition or activation of δPKC.

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Background of the Invention

Protein kinase C (PKC) is a key enzyme in signal transduction involved in a variety of cellular functions, including cell growth, regulation of gene expression and ion channel activity. The PKC family of isozymes includes at least 11 different protein kinases which can be divided into at least three subfamilies based on their homology and sensitivity to activators. Members of the classical or cPKC subfamily, α, β_I, β_{II} and γPKC, contain four homologous domains (C1, C2, C3 and C4) inter-spaced with isozyme-unique (variable or V) regions, and require calcium, phosphatidylserine (PS), and diacylglycerol (DG) or phorbol esters for activation. Members of the novel or nPKC subfamily, δ, ε, η, and θPKC, lack the C2 homologous domain and do not require calcium for activation. Finally, members of the atypical or αPKC subfamily, ζ and λ/ιPKC, lack both the C2 and one half of the C1 homologous domains and are insensitive to DG, phorbol esters and calcium.

Studies on the subcellular distribution of PKC isozymes demonstrate that activation of PKC results in its redistribution in the cells (also termed translocation), such that activated PKC isozymes associate with the plasma membrane, cytoskeletal elements, nuclei, and other subcellular compartments (Saito, *et al.*, 1989; Papadopoulos and Hall, 1989; Mochly-Rosen, *et al.*, 1990).

It appears that the unique cellular functions of different PKC isozymes are determined by their subcellular location. For example, activated β_IPKC is found inside the nucleus, whereas activated β_{II}PKC is found at the perinucleus and cell periphery of cardiac myocytes (Disatnik, *et al.*, 1994). Further, in the same cells, εPKC binds to cross-striated structures (possibly the contractile elements) and cell-cell contacts following activation or after addition of exogenous activated εPKC to fixed cells (Mochly-Rosen, *et al.*, 1990; Disatnik, *et al.*, 1994). The localization of different PKC isozymes to different areas of the cell in turn appears due to binding of the activated isozymes to specific anchoring molecules termed Receptors for Activated C-Kinase (RACKs).

RACKs are thought to function by selectively anchoring activated PKC isozymes to their respective subcellular sites. RACKs bind only fully activated PKC and are not necessarily substrates of the enzyme. Nor is the binding to RACKs mediated via the catalytic domain of the kinase (Mochly-Rosen, *et al.*, 1991). Translocation of PKC reflects

binding of the activated enzyme to RACKs anchored to the cell particulate fraction and the binding to RACKs is required for PKC to produce its cellular responses (Mochly-Rosen, 1995). Inhibition of PKC binding to RACKs *in vivo* inhibits PKC translocation and PKC-mediated function (Johnson, *et al.*, 1996a; Ron, *et al.*, 1995; Smith and Mochly-Rosen, 1992).

cDNA clones encoding RACK1 and RACK2 have been identified (U.S. Patent 5,519,003; Ron, *et al.*, 1994; Csukai, *et al.*, 1995). Both are homologs of the β subunit of G proteins, a receptor for another translocating protein kinase, the β-adrenergic receptor kinase, βARK (Pitcher, *et al.*, 1992). Similar to Gβ, RACK1, and RACK2 have seven WD40 repeats (Ron, *et al.*, 1994; Csukai, *et al.*, 1995). Recent data suggest that RACK1 is a β_{II}PKC-specific RACK (Stebbins *et al.,* 2001) and that RACK2 (Csukai *et al.*, 1997) is specific for activated εPKC.

Translocation of PKC is required for proper function of PKC isozymes. Peptides that mimic either the PKC-binding site on RACKs (Mochly-Rosen *et al.*, 1991a; Mochly-Rosen *et al.*, 1995) or the RACK-binding site on PKC (Ron, *et al.*, 1995; Johnson, *et al.*, 1996a) are isozyme-specific translocation inhibitors of PKC that selectively inhibit the function of the enzyme *in vivo*. For example, an eight amino acid peptide derived from εPKC (peptide εV1-2; SEQ ID NO:1, Glu Ala Val Ser Leu Lys Pro Thr) is described in U.S. Patent No. 6,165,977. The peptide contains a part of the RACK-binding site on εPKC and selectively inhibits specific εPKC-mediated functions in cardiac myocytes. This εPCK peptide has been shown to be involved in cardiac preconditioning to provide protection from ischemic injury. Prolonged ischemia causes irreversible myocardium damage primarily due to death of cells at the infarct site. Studies in animal models, isolated heart preparations and isolated cardiac myocytes in culture have demonstrated that short bouts of ischemia of cardiac muscle reduce such tissue damage in subsequent prolonged ischemia (Liu, Y., *et al.*, 1995, 1996; Hu, *et al.*, 1995; Brew, *et al.*, 1995; Schultz, *et al.*, 1996). This protection, which occurs naturally following angina and has been termed preconditioning, can be mimicked by a variety of non-specific PKC agonists (Mitchell, *et al.*, 1993; Mitchell, *et al.*, 1995; Murry, *et al.*, 1986; Speechly-Dick, *et al.*, 1994). Both δPKC and εPKC activation occurs following preconditioning (Gray *et al.*, 1997), however, εPKC activation is required for protection of cardiac myocytes from ischemia-induced cell death (U.S. Patent No. 6,165,977).

In a recent study, an εPKC-selective peptide agonist was shown to provide cardio-protection from ischemia when administered intracellulary to isolated neonatal and adult

cardiomyocytes and when produced intracellulary *in vivo* in transgenic mice (Dorn G. *et al.*, 1999).

The ability of δPCK peptide agonists and antagonists to protect cells and tissue from an ischemic event or to reverse or reduce damage caused by an ischemic event has not been reported. More particularly, it is unknown in the art whether or not δPCK peptide agonists and antagonists can be delivered extracellulary to whole tissue or intact organs *in vivo* to achieve a therapeutic effect.

Summary of the Invention

Accordingly, it is an object of the invention to provide a method of protecting tissue from damage due to an ischemic event.

It is a further object of the invention to provide a method of administering an δPKC peptide antagonist for protection of cells and tissue from damage due to an ischemic event.

It is yet another object of the invention to provide a method of ameliorating damage to tissue caused by an ischemic event.

It is still a further objective of the invention to provide a method of reducing or protecting cells and tissue from damage as a result of stroke.

It is another objective of the invention to provide a method of enhancing cellular or tissue damage as a result of an ischemic or hypoxic event.

In one aspect, the invention includes a peptide selected δV1-1 (SEQ ID NO:4), δV1-2 (SEQ ID NO:5), ψδRACK (SEQ ID NO:6), δV1-5 (SEQ ID NO:7), and derivatives and fragments thereof. Exemplary derivatives of δV1-1 are identified as SEQ ID NOS:34-48. Exemplary derivatives of δV1-2 are identified as SEQ ID NOS:65-71. Exemplary derivatives of ψδRACK are identified as SEQ ID NOS:11-19, 22-33. Exemplary fragments of δV1-1 are identified as SEQ ID NOS:49-64. Exemplary fragments of ψδRACK are identified as SEQ ID NO:20 and SEQ ID NO:21.

In one embodiment, the peptide is recombinantly produced, such as where the peptide is encoded by a polynucleotide. In other embodiments, the peptide is chemically synthesized.

In one embodiment, the peptide is linked to a moiety effective to facilitate transport across a cell membrane. Exemplary moieties include a Tat-derived peptide, an Antennapedia carrier peptide, and a polyarginine peptide.

In another embodiment, the peptide is joined to a second peptide to form a fusion peptide.

In another aspect, the invention includes a method of reducing ischemic injury to a cell or a tissue exposed to hypoxic conditions by administering to the cell or tissue an amount of an isozyme-specific δPKC antagonist. Contemplated antagonists include δV1-1 (SEQ ID NO:4), δV1-2 (SEQ ID NO:5), δV1-5 (SEQ ID NO:7), and derivatives and fragments thereof.

In various embodiments of this method, the peptide is administered prior to, during or after exposing the cell or tissue to said hypoxic conditions. The peptide can be linked to a carrier peptide, as described above.

In one embodiment, the peptide is administered by infusion through coronary arteries to an intact heart.

In another aspect, the invention includes a method of reducing or preventing or ameliorating damage to a cell or tissue due to stroke by administering to the cell or tissue an amount of an isozyme-specific δPKC antagonist. Contemplated antagonists include δV1-1 (SEQ ID NO:4), δV1-2 (SEQ ID NO:5), δV1-5 (SEQ ID NO:7), and derivatives and fragments thereof.

In various embodiments of this method, the peptide is administered prior to, during or after the stoke, when the cell or tissue is exposed to a hypoxic event. The peptide can be linked to a carrier peptide, as described above.

In another aspect, the invention includes a method of enhancing damage to a cell exposed to hypoxic conditions by administering to the cell an amount of an isozyme-specific δPKC agonist. Contemplated agonists include ψδRACK identified as SEQ ID NO:6, derivatives and fragments or ψδRACK. Exemplary derivatives include peptides identified as SEQ ID NOS:11-19, and SEQ ID NOS:22-29. Exemplary fragments include the peptides identified as SEQ ID NOS:20-21.

In one embodiment, the peptide is administered to a tumor cell. The agonist peptide can be linked to a moiety effective to facilitate transport across a cell membrane.

In another aspect, the invention includes a method of identifying a compound effective to induce protection of a cell from hypoxic or ischemic damage. In the method, a δPKC peptide containing a δRACK binding site is contacted with a δPKC antagonist peptide with the δRACK binding site in the presence and absence of said test compound. The test compound is identified as being effective to induce protection if (i) binding in the

presence of the test compound is decreased relative to binding in the absence of the test compound, or (ii) catalytic activity of the test compound is increased relative to activity in the absence of the test compound.

In this method, the δPKC peptide can be selected from the group consisting of δV1-1 (SEQ ID NO:4), δV1-2 (SEQ ID NO:5), δV1-5 (SEQ ID NO:7), and fragments and derivatives thereof.

In another aspect, the invention includes a method of identifying a compound effective to enhance hypoxic or ischemic damage in a cell. A ψδRACK agonsit peptide is contacted with a δPKC peptide containing a RACK binding site in the presence and absence of a test compound. The test compound is identified as being effective to enhance ischemic damage if (i) binding in the presence of the test compound is decreased relative to binding in the absence of the test compound, or (ii) the catalytic activity of the δPKC in the presence of the test compound is increased relative to the catalytic activity in the absence of the test compound.

In one embodiment, a ψδRACK peptide selected from the group consisting of SEQ ID NO:6, fragments, and derivatives thereof is used. Exemplary suitable derivatives and fragments are identified in SEQ ID NOS:11-29.

In another aspect, the invention includes a method of providing protection to tissue from damage caused by an ischemic or hypoxic event by administering to the tissue a peptide selected from the group consisting of SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:7, derivatives and fragments thereof. Suitable derivatives and fragments include those given above.

In one embodiment, the peptide is administered by the intraveneous, parenteral, subcutaneous, inhalation, intranasal, sublingual, mucosal, and transdermal route. In another method, the peptide is admistered during a period of reperfusion; that is, after a period of initial perfusion.

Protection against ischemia is provided to a variety of tissues, including but not limited to the brain, heart, eye, and kidney.

These and other objects and features of the invention will be more fully appreciated when the following detailed description of the invention is read in conjunction with the accompanying drawings.

Brief Description of the Drawings

Fig. 1 shows the alignment of the primary sequence of rat δPKC and mouse ΘPKC V1 domains. The bracketed areas designated as δV1-1, δV1-2, and ψδR indicate regions of difference between the two isozymes.

Fig. 2A shows a Western blot autoradiogram of soluble (S) and particulate (P) cell fractions after treatment with δV1-1 in the presence and absence of phorbol 12-myristate 13-acetate (PMA) and probing with anti-δPKC and anti-εPKC antibodies.

Fig. 2B shows the translocation of δPKC and εPKC, expressed as the amount of isozyme in the particulate fraction over the amount of isozyme in non-treated cells, for cells treated as indicated in Fig. 2A with δV1-1 in the presence (+) and absence (-) of PMA.

Fig. 3A shows a Western blot autoradiogram of soluble (S) and particulate (P) cell fractions after treatment with ψδRACK or with PMA and probing with anti-δPKC and anti-αPKC antibodies.

Fig. 3B shows the translocation of δPKC and αPKC, expressed as the amount of isozyme in the particulate fraction over the amount of isozyme in non-treated cells, for cells treated as indicated in Fig. 3A with ψδRACK in the presence (+) and absence (-) of PMA.

Fig. 4A shows a Western blot autoradiogram of soluble (S) and particulate (P) cell fractions after treatment with δV-1 in the presence and absence of ψδRACK and probing with anti-δPKC and anti-εPKC antibodies.

Fig. 4B shows the translocation of δPKC, expressed as the amount of isozyme in the particulate fraction over the amount of isozyme in non-treated cells, for cells treated as indicated in Fig. 4A with δV1-1 in the presence (+) and absence (-) of ψδRACK.

Fig. 5A shows percentage of cell damage for isolated cardiac myocytes treated with δV1-1 in the absence (-) or presence (in the concentrations indicated along the x-axis) of ψδRACK . The peptides were administered 10 minutes prior to a 180 minute ischemic period. As a control, βPKC-selective activator peptide was used.

Fig. 5B shows percentage of cell damage for isolated cardiac myocytes treated with δV1-1 in the absence (-) or presence (in the concentrations indicated along the x-axis). The peptides were administered 10 minutes prior to a 90 minute ischemic period.

Fig. 6A shows the cell damage, as measured by creatine phosphokinase (CPK) release in whole rat hearts treated *ex vivo* with δV1-1 (solid circles) or with ψδRACK

(solid diamonds) as a function of time post-ischemia and post-treatment. As controls, some hearts were left untreated prior to ischemia (open squares) and other hearts were maintained in normoxia conditions (open triangles).

Fig. 6B is a bar graph showing the total cell damage, as measured by total CPK release for the *ex vivo* hearts treated as described in Fig. 6A with δV1-1 and with ψδRACK, as well as *ex vivo* hearts treated with two controls: the Tat-carrier peptide alone and with a scrambled δV1-1 sequence conjugated to Tat-carrier peptide.

Figs. 7A-7B show the functional recovery of a working heart perfused with δV1-1 (Fig. 7A) or left untreated (Fig. 7B) after 20 minutes of global ischemia, where the left ventricle developed pressure (LVP, in mmHg), its first derivative (*dP/dt*, in mmHg/sec), and the coronary perfusion pressure (PP, in mmHg) are shown. On the right, an expanded trace of the same functional measurement are shown before (base line) and 30 minutes after reperfusion.

Figs. 8A-8C are plots of percent of left ventricular developed pressure (%LVDP, Fig. 8A), end diastolic pressure (EDP, Fig. 8B) and perfusion pressure (PP, Fig. 8C) of a working heart as a function of time before ischemia (baseline) and 5 to 30 minutes after ischemia and during treatement δV1-1 (closed squares) or untreated (open circles).

Figs. 9A-9B are photos obtained by a digital camera of pig heart slices taken from the pigs five days after treatment *in vivo* with δV1-1 (Fig. 9A) or with the carrier peptide alone as a control (Fig. 9B) during the last 10 minutes of a 30 minute ischemic insult.

Fig. 9C is a bar graph showing the percent of infarct of the area at risk determined from the heart slices of Figs. 9A-9B, for the pigs treated with δV1-1 and for the untreated, control animals.

Fig. 10 is a graph showing the ejection fraction, as measured by left ventricurogram in pigs at three time points: (1) before occlusion of left anterior descending artery by balloon catheter (pre ischemia); (2) immediately after reperfusion with δV1-1 (post ischemia); and (3) before sacrifice five days later (5 days post ischemia), for animals treated with δV1-1 (solid circles) and for control animals treated with a scrambled peptide (open circles).

Figs. 11A-11B are digitized photographs of brains taken from untreated animals (Fig. 11A) and animals treated with δV1-1 (Fig. 11B) prior to an induced stroke.

Brief Description of the Sequences

SEQ ID NO:1 is an eight amino acid peptide derived from εPKC, referred to as εV1-2 and described in U.S. Patent No. 6,165,977.

SEQ ID NO:2 corresponds to amino acids 1-141 from the V1 domain of rat δPKC (accession no. KIRTCD).

SEQ ID NO:3 corresponds to amino acids 1-124 of V1 domain of mouse θPKC (accession no. Q02111).

SEQ ID NO:4 is an amino acid sequence from the first variable region of δPKC (amino acids 8-17), δV1-1.

SEQ ID NO:5 is an amino acid sequence from the first variable region of δPKC (amino acids 35-44), δV1-2.

SEQ ID NO:6 is an amino acid sequence from δPKC (amino acids 74-81), and is referred to herein as "pseudo-delta" RACK, or ψδRACK.

SEQ ID NO:7 is an amino acid sequence from a region of δPKC (amino acids 619-676), referred to herein as δV1-5.

SEQ ID NO:8 is the *Drosophila* Antennapedia homeodomain-derived carrier peptide.

SEQ ID NO:9 is a Tat-derived carrier peptide (Tat 47-57).

SEQ ID NO:10 is a βPKC-selective activator peptide.

SEQ ID NO:11 is a modification of SEQ ID:NO 6 (ψδRACK).

SEQ ID NO:12 is a modification of SEQ ID:NO 6 (ψδRACK).

SEQ ID NO:13 is a modification of SEQ ID:NO 6 (ψδRACK).

SEQ ID NO:14 is a modification of SEQ ID:NO 6 (ψδRACK).

SEQ ID NO:15 is a modification of SEQ ID:NO 6 (ψδRACK).

SEQ ID NO:16 is a modification of SEQ ID:NO 6 (ψδRACK).

SEQ ID NO:17 is a modification of SEQ ID:NO 6 (ψδRACK).

SEQ ID NO:18 is a modification of SEQ ID:NO 6 (ψδRACK).

SEQ ID NO:19 is a modification of SEQ ID:NO 6 (ψδRACK).

SEQ ID NO:20 is a fragment of SEQ ID:NO 6 (ψδRACK).

SEQ ID NO:21 is a fragment of SEQ ID:NO 6 (ψδRACK).

SEQ ID NO:22 is a modification of SEQ ID:NO 6 (ψδRACK).

SEQ ID NO:23 is a modification of SEQ ID:NO 6 (ψδRACK).

SEQ ID NO:24 is a modification of SEQ ID:NO 6 (ψδRACK).

SEQ ID NO:25 is a modification of SEQ ID:NO 6 (ψδRACK).

SEQ ID NO:26 is a modification of SEQ ID:NO 6 (ψδRACK).

SEQ ID NO:27 is a modification of SEQ ID:NO 6 (ψδRACK).

SEQ ID NO:28 is a modification of SEQ ID:NO 6 (ψδRACK).

SEQ ID NO:29 is a modification of SEQ ID:NO 6 (ψδRACK).

SEQ ID NO:30 is a modification of SEQ ID:NO 6 (ψδRACK).

SEQ ID NO:31 is a modification of SEQ ID:NO 6 (ψδRACK).

SEQ ID NO:32 is a modification of SEQ ID:NO 6 (ψδRACK).

SEQ ID NO:33 is a modification of SEQ ID:NO 6 (ψδRACK).

SEQ ID NO:34 is a modification of SEQ ID:NO 4 (δV1-1).

SEQ ID NO:35 is a modification of SEQ ID:NO 4 (δV1-1).

SEQ ID NO:36 is a modification of SEQ ID:NO 4 (δV1-1).

SEQ ID NO:37 is a modification of SEQ ID:NO 4 (δV1-1).

SEQ ID NO:38 is a modification of SEQ ID:NO 4 (δV1-1).

SEQ ID NO:39 is a modification of SEQ ID:NO 4 (δV1-1).

SEQ ID NO:40 is a modification of SEQ ID:NO 4 (δV1-1).

SEQ ID NO:41 is a modification of SEQ ID:NO 4 (δV1-1).

SEQ ID NO:42 is a modification of SEQ ID:NO 4 (δV1-1).

SEQ ID NO:43 is a modification of SEQ ID:NO 4 (δV1-1).

SEQ ID NO:44 is a modification of SEQ ID:NO 4 (δV1-1).

SEQ ID NO:45 is a modification of SEQ ID:NO 4 (δV1-1).

SEQ ID NO:46 is a modification of SEQ ID:NO 4 (δV1-1).

SEQ ID NO:47 is a modification of SEQ ID:NO 4 (δV1-1).

SEQ ID NO:48 is a modification of SEQ ID:NO 4 (δV1-1).

SEQ ID NO:49 is a fragment of SEQ ID:NO 4 (δV1-1).

SEQ ID NO:50 is a modified fragment of SEQ ID:NO 4 (δV1-1).

SEQ ID NO:51 is a modified fragment of SEQ ID:NO 4 (δV1-1).

SEQ ID NO:52 is a modified fragment of SEQ ID:NO 4 (δV1-1).

SEQ ID NO:53 is a modified fragment of SEQ ID:NO 4 (δV1-1).

SEQ ID NO:54 is a modified fragment of SEQ ID:NO 4 (δV1-1).

SEQ ID NO:55 is a modified fragment of SEQ ID:NO 4 (δV1-1).

SEQ ID NO:56 is a modified fragment of SEQ ID:NO 4 (δV1-1).

SEQ ID NO:57 is a modified fragment of SEQ ID:NO 4 (δV1-1).

SEQ ID NO:58 is a fragment of δV1-1.

SEQ ID NO:59 is a fragment of δV1-1.

SEQ ID NO:60 is a fragment of δV1-1.

SEQ ID NO:61 is a fragment of δV1-1.

SEQ ID NO:62 is a fragment of δV1-1.

SEQ ID NO:63 is a fragment of δV1-1.

SEQ ID NO:64 is a fragment of δV1-1.

SEQ ID NO:65 is a modification of SEQ ID:NO 5 (δV1-2).

SEQ ID NO:66 is a modification of SEQ ID:NO 5 (δV1-2).

SEQ ID NO:67 is a modification of SEQ ID:NO 5 (δV1-2).

SEQ ID NO:68 is a modification of SEQ ID:NO 5 (δV1-2).

SEQ ID NO:69 is a modification of SEQ ID:NO 5 (δV1-2).

SEQ ID NO:70 is a modification of SEQ ID:NO 5 (δV1-2).

SEQ ID NO:71 is a modification of SEQ ID:NO 5 (δV1-2).

SEQ ID NO:72 is the sequence of Annexin V.

Detailed Description of the Invention

I. Definitions

Unless otherwise indicated, all terms herein have the same meaning as they would to one skilled in the art of the present invention. Practitioners are particularly directed to *Current Protocols in Molecular Biology* (Ausubel, F. M. *et al.*, John Wiley and Sons, Inc., Media Pa.) for definitions and terms of the art.

Abbreviations for amino acid residues are the standard 3-letter and/or 1-letter codes used in the art to refer to one of the 20 common L-amino acids.

A "conserved set" of amino acids refers to a contiguous sequence of amino acids that is conserved between members of a group of proteins. A conserved set may be anywhere from two to over 50 amino acid residues in length. Typically, a conserved set is between two and ten contiguous residues in length. For example, for the two peptides MKAAEDPM (SEQ ID NO:11) and MRAPEDPM (SEQ ID NO:14), there are 4 identical positions (EDPM; SEQ ID NO:20) which form the conserved set of amino acids for these two sequences.

"Conservative amino acid substitutions" are substitutions which do not result in a significant change in the activity (*e.g.*, δV1-1 PKC activity) or tertiary structure of a selected polypeptide or protein. Such substitutions typically involve replacing a selected amino acid residue with a different residue having similar physico-chemical properties. For example, substitution of Glu for Asp is considered a conservative substitution since both are similarly-sized negatively-charged amino acids. Groupings of amino acids by physico-chemical properties are known to those of skill in the art.

"Peptide" and "polypeptide" are used interchangeably herein and refer to a compound made up of a chain of amino acid residues linked by peptide bonds. Unless otherwise indicated, the sequence for peptides is given in the order from the amino termiums to the carboxyl terminus.

Two amino acid sequences or two nucleotide sequences are considered homologous (as this term is preferably used in this specification) if they have an alignment score of >5 (in standard deviation units) using the program ALIGN with the mutation gap matrix and a gap penalty of 6 or greater (Dayhoff, M. O., in ATLAS OF PROTEIN SEQUENCE AND STRUCTURE (1972) Vol. 5, National Biomedical Research Foundation, pp. 101-110, and Supplement 2 to this volume, pp. 1-10.) The two sequences (or parts thereof) are more preferably homologous if their amino acids are greater than or equal to 50%, more preferably 70%, still more preferably 80%, identical when optimally aligned using the ALIGN program mentioned above.

A peptide or peptide fragment is "derived from" a parent peptide or polypeptide if it has an amino acid sequence that is identical or homologous to the amino acid sequence of the parent peptide or polypeptide.

"Ischemia" or an "ischemic event" refers to an insufficient supply of blood to a specific cell, tissue or organ. A consequence of decreased blood supply is an inadequate supply of oxygen to the organ or tissue (hypoxia). Prolonged hypoxia may result in injury to the affected organ or tissue.

"Anoxia" refers to a virtually complete absence of oxygen in the organ or tissue, which, if prolonged, may result in death of the cell, organ or tissue.

"Hypoxia" or a "hypoxic condition" intend a condition under which a cell, organ or tissue receive an inadequate supply of oxygen.

"Reperfusion"refers to return of fluid flow into a tissue after a period of no-flow or reduced flow. For example, in reperfusion of the heart, fluid or blood retursn to the heart through the coronary arteries after occlusion of these arteries has been removed.

"Tissue" as used herein intends a whole organ, either in vivo or ex vivo, a fragment of an organ, or two or more cells.

The term "PKC" refers to protein kinase C, or C-kinase.

The term "RACK" refers to receptor for activated C-kinase.

II. δPKC Peptide Agonists and Antagonists

In one aspect, the invention includes peptides effective to activate δPKC and peptides effective to inhibit δPKC. The sequence of the RACK for δPKC is unknown as this RACK has not yet been identified. Thus, it is a challenge to identify δPKC-selective activator and inhibitor peptides in the absence of any information about the δRACK sequence. Further, the exact role of δPKC in response to ischemia is also not known in the art. It is known that δPKC, like εPKC, undergoes translocation on ischemic preconditioning in rat (Gray, M.O. *et al.*; Chen, C.-H. *et al.*). However, whether the δPKC translocation results in protection from ischemia or not has been unknown until the present invention.

In studies performed in support of the present invention to identify peptide sequences for activation and inhibition of δPKC, the sequence of δPKC was compared to the sequence of θPKC, since of the three other novel PKC isozymes, δPKC is most similar to θPKC with a 52% identity of amino acid sequence (Osada, S.-I *et al.*; Baier, G. *et al.*). It was also assumed that each PKC isozyme should interact with a different RACK. Since the first variable (V1) domain of δPKC contains the RACK-binding site (Johnson *et al.* 1996a) regions least similar to θPKC may be involved in RACK binding. Fig. 1 compares the sequences of the V1 domain of rat δPKC (SEQ ID NO:2; accession no. KIRTCD) and mouse θPKC V1 domain (SEQ ID NO:3, accession no. Q02111). Three regions in the V1 domain of δPKC were identified with only ~10% identity to θPKC. These regions are indicated in Fig. 1 by the bars above the sequence of δPKC and are referred to herein as δV1-1 having a sequence identified herein as SEQ ID NO:4 (SFNSYELGSL), δV1-2 having a sequence identified herein as SEQ ID NO:5 (ALTTDRGKLV), and ψδRACK having a sequence identified herein as SEQ ID NO:6 (MRAAEDPM). Not shown in Fig. 1 is yet another sequence identified from the δPKC sequence for testing of its activation or inhibition of δPKC. This sequences is identified as SEQ ID NO7 and is referred to herein as δV1-5.

As described in Example 1, the δV1-1 and ψδRACK peptides were analyzed to determine whether the peptides had activity, and if so, whether the activity was as an

agonist or an antagonist of δPKC. As will be shown, δV1-1, δV1-2 and δV1-5 are δPKC antagonists and ψδRACK is a δPKC agonist. In these studies, the δV1-1 and ψδRACK peptides were modified with a carrier peptide by cross-linking via an N-terminal Cys-Cys bond to the *Drosophila* Antennapedia homeodomain (SEQ ID NO:8; Théodore, L., *et al.*; Johnson, J. A. *et al.*, 1996b). In other studies, not described here, the peptide was modified with Tat (SEQ ID NO:9) or with polyarginine (Mitchell *et al.*, 2000; Rolhbard *et al.*, 2000) and gave results similar to those described herein. Details of the study are set forth in Example 1. In brief, the Antennapedia-conjugated peptides were introduced to cardiac cells at a concentration of 500 nM in the presence and absence of phorbol 12-myristate 13-acetate (PMA) or in the presence of each other. Translocation of δPKC isozyme was assessed by Western blot analysis cystosolic and particulate fractions of treated cells. Subcellular localization of δPKC isozyme was assessed by immunofluorescence by probing the blot with anti-δPKC, anti-αPKC, and anti-ε PKC antibodies. Translocation was expressed as the amount of isozyme in the particulate fraction over the amount of isozyme in non-treated cells. The results are shown in Figs. 2-4.

Figs. 2A-2B show the results for the cells treated with δV1-1 in the presence (+) and absence (-) of PMA. Fig. 2A is the Western blot autoradiogram of soluble (S) and particulate (P) cell fractions after treatment with the peptide and after probing with anti-δPKC and anti-εPKC antibodies. Fig. 2B shows the translocation of δPKC expressed as the amount of isozyme in the particulate fraction over the amount of isozyme in non-treated cells. The δV1-1 peptide inhibited PMA-induced δPKC translocation. In other studies no shown here, the δV1-1 peptide did not inhibit the translocation of εPKC or αPKC.

Figs. 3A-3B are similar plots for the cells treated with ψδRACK in the presence (+) and absence (-) of PMA. ψδRACK was opposite in effect from δV1-1 in that it selectively induced δPKC translocation in cardiac myocytes, without affecting the translocation of PKCα or εPKC (not shown).

Figs. 4A-4B shows the results for the cells treated with δV1-1 in the presence and absence of ψδRACK. Basal partitioning of δPKC in the particulate fraction was inhibited by δV1-1 and the presence of ψδRACK reversed this δV1-1 effect.

Together the results in Figs. 2-4 shows that δV1-1 is a selective translocation inhibitor of δPKC and that ψδRACK is analogous to the ψRACK site and acts as a selective translocation activator of δPKC.

A. Protection of Cells from Damage Due to Ischemia

In another study, the δPKC activator peptide, ψδRACK, and the δPKC inhibitor peptide, δV1-1 were administered to isolated rat caridac myocytes to determine the role of δPKC in protection from ischemia. As described in Example 2, the Antennapedia carrier-peptide conjugate of δV1-1 and/or ψδRACK was introduced into isolated adult rat cardiac myocytes ten minutes prior to prolonged ischemia. Cell damage was assessed using an osmotic fragility test by measuring uptake of trypan blue. The results are shown in Figs. 5A-5C.

Fig. 5A shows the results for cells treated with δV1-1 at concentrations of 10 nM, 100 nM, 500 nM, and 1 μM in the presence or absence (-) of 1 μM ψδRACK. The results are presented as the percentage of cell damage for cells treated as indicated along the x-axis. As a control, a βPKC-selective activator peptide (SVEIWD, SEQ ID NO:10) was used. The peptides were administered ten minutes prior to the 180 minute ischemic period. The presence of δV1-1 administered prior to ischemia resulted in a concentration-dependent level of protection from ischemia-induced damage. The protection was prevented by co-incubation with the δPKC-specific translocation activator peptide, ψδRACK, but not with co-incubation with the control βPKC-selective translocation activator.

The data in Fig. 5A suggested that activation of δPKC with ψδRACK caused a slight increase in cardiac myocyte damage after an ischemic insult. Based on this, ψδRACK was hypothesized as acting synergistically with ischemia-induced activation of δPKC to cause cell damage. This was evaluated by reducing the period of ischemic insult, since synergism between ψδRACK and ischemia in inducing cell damage should become apparent when ischemic insult was reduced. Thus, another study was performed where the ischemic period was shortened to 90 minutes. The results of this study are shown in Fig. 5B. The ψδRACK-induced increase in cell damage became significant when the time of ischemia was shortened from 180 to 90 minutes, and was reversed by co-treatment with the δPKC inhibitor, δV1-1. Therefore, activation of δPKC by ischemia appears to mediate cell damage. Together, Figs. 5A and 5B demonstrate that cell damage induced by simulated ischemia is due, at least in part, to activation of δPKC.

B. *Ex vivo* Delivery of Peptides to Whole Hearts

In another study performed in support of the invention, the δPKC selective inhibitor peptide, δV1-1, or the activator peptide, ψδRACK, were delivered to whole hearts *ex vivo* to determine if the peptides have activity when introduced extracellulary to a whole organ. As described in Example 3, δV1-1 and ψδRACK peptides were conjugated to a carrier peptide, a Tat-derived peptide. The peptides were delivered into Langendorff perfused rat hearts prior to induction of an ischemic period. After perfusion with the peptides, global ischemia was effected for 30 minutes. After the 30 minute ischemic period, the amount of creatine phosphokinase (CPK) released was monitored during a 30 minute reperfusion period. The results are shown in Figs. 6A-6B.

Fig. 6A shows the cell damage, as measured by creatine phosphokinase (CPK) release in the whole rat hearts treated with δV1-1 (solid circles) or with ψδRACK solid diamonds) as a function of time during the post-ischemia, reperfusion period. As controls, some hearts were left untreated prior to ischemia (open squares) and other hearts were maintained in normoxia conditions (open triangles).

Fig. 6B is a bar graph showing the total cell damage, as measured by total CPK release for the *ex vivo* hearts treated as described in Fig. 6A with δV1-1 and ψδRACK. Fig. 6B also shows the total cell damage for *ex vivo* hearts treated with two controls: the Tat-carrier peptide alone and with a scrambled ψδRACK sequence conjugated to Tat-carrier peptide.

Figs. 6A-6B show that acute administration of the δPKC activator, ψδRACK, enhanced cardiac damage induced by ischemia by about 30%. Acute administration of the δPKC-selective inhibitor, δV1-1, protected hearts against ischemic damage as shown by decreased release of creatine kinase. Together, these data indicate that in an intact heart, inhibition of δPKC conferred greater than 50% protection against ischemic damage (Fig. 6A). Accordingly, the invention contemplates a method of protecting a cell or a tissue from damage due to ischemia by administering a δPKC-selective antagonist, such as δV1-1, δV1-2, δV1-5, to the tissue. Such administration is effective to reduce cell damage by at least about 10%, more preferably by at least about 25%, and most preferably by at least about 50% when compared to tissue left untreated prior to an ischemic insult.

Another study was performed to determine if the peptides could be delivered to an intact organ to provide protection after an ischemic insult. In this study, as described in Example 4, the rat heart model described above was used and the hemodynamic parameters

were measured during the 20 minutes of global ischemia and the 20 minutes of reperfusion. During the reperfusion only, δV1-1 was delivered at a concentration of 500 nM. The results are shown in Figs. 7A-7B.

Fig. 7A shows the functional recovery of a working heart perfused with δV1-1 after 20 minutes of global ischemia, where the left ventricle developed pressure (LVP, in mmHg), its first derivative (*dP/dt*, in mmHg/sec), and the coronary perfusion pressure (PP, in mmHg) are shown. Fig. 7B is a similar plot for an untreated heart. As seen by comparing the traces for the δV1-1 treated heart (Fig. 7A) and the untreated heart Fig. 7B), when δV1-1 was delivered during the first 20 minutes of reperfusion, there was a significant improvement in functional recovery after ischemia. In particular, a significant improvement in both the LVP recovery and its first derivative (*dP/dt*) were achieved by administering δV1-1 after ischemic insult. The δV1-1 peptide reduced the elevated LVP end diastolic pressure and the coronary perfusion pressure (PP). In addition there was a ~50% reduction in creatine phosphokinase release as compared with hearts treated with vehicle control (not shown).

In a similar study, five pairs of rats were treated as described in Example 4, where the *ex vivo* hearts were subjected to 20 minutes of ischemia and 30 minutes of reperfusion. During the first 20 minutes of reperfusion, 500 nM of δV1-1 or vehicle control was administered. The averaged results are shown in Figs. 8A-8C.

Fig. 8A shows the percent of left ventricular developed pressure (%LVDP) before ischemia, noted on the x-axis as "baseline" and during the 5-30 minute period after reperfusion was provided. Data were collected during the reperfusion, meaning during and after treatment with δV1-1. Hearts treated with the δV1-1 peptide (closed squares) had a 2-fold to 4-fold higher LVDP than hearts left untreated (open circles).

Fig. 8B is a similar plot showing the end diastolic pressure (EDP) before ischemia, noted as "baseline" on the x-axis, and during the 5-30 minute period after reperfusion treatment with δV1-1 (closed squares) or after reperfusion with a control vehicle (open circles). The EDP for hearts treated with δV1-1 was approximately 60 mmHg. Hearts left untreated after ischemia (open circles) had an EDP of between about 70-80 mmHg.

Fig. 8C shows the perfusion pressure (PP) of the δV1-1 treated hearts (closed squares) and the untreated hearts (open circles). The baseline perfusion pressure before ischemia is indicated on the x-axis. After ischemia and after treatment with δV1-1 the perfusion pressure was about 75% of that found hearts left untreated.

The data in Figs. 7-8 show that administration of a δPKC antagonist peptide, such as δV1-1, δV1-2, δV1-5, after an ischemic insult to a cell or tissue is effective to protect the cell or tissue from damage du to ischemia and resulting hypoxia. The data also show that a δPKC antagonist peptide is effective to reduce or minimize the damage due to ischemia and hypoxia allowing the tissue to recover its functional properties following ischemia.

C. *In vivo* Treatment with δV1-1

In another study in support of the invention, the ability of δV1-1 peptide to protect tissue from damage due to an ischemic or hypoxic event was evaluated by administering the peptide *in vivo* to adult female pigs. As detailed in Example 5, δV1-1 peptide was administered to the pigs during the last 10 minutes of a 30 minute ischemic insult. Five days later, the hearts were analyzed for tissue damage. The results are shown in Figs. 9A-9B.

Figs. 9A-9B are digitized photos of pig heart slices taken from the pigs treated *in vivo* five days earlier with δV1-1 (Fig. 9A) or with the carrier peptide alone as a control (Fig. 9B). The hearts were stained with a double-staining technique (Example 5) that allowed determination of the area at risk for ischemic injury (area within the arrows, mainly in the lower hemisphere between the two arrows) and infarcted area (white area in Fig. 9B). As seen in Fig. 9B, control hearts have a large infarct area within the area at risk (borders shown with arrows). In contrast, pigs that received the δV1-1 peptide (Fig. 9A) have a significantly reduced infarct area. The white area in Fig. 9A that is outside the area of risk (outside the arrows) is connective tissue and fat, and is not an infarcted area.

Fig. 9C is a bar graph showing the percent of infarct of the area at risk for the untreated, control animals and the animals treated with δV1-1. Animals treated with a δPKC antagonist had a nearly two-fold lower percentage of infarct than animals left untreated. Together, Figs. 9A-9C show that δV1-1 can be administered *in vivo* to a whole organ and provide protection from damage due to ischemia.

Blood samples and tissue samples of lung, liver, brain, gut, kidney, etc. were collected from the animals and analyzed at a pathology lab. All samples were normal and no inflammation or tissue abnormalities were observed. In addition, there was no adverse effect of two injections of the δV1-1 antagonist peptide at 1μM final concentration in the mouse model. Kidney, liver, brain, and lung functions were normal and all blood analyses were also normal.

In another study, left ventricurogram was performed in pigs (n=5) at three time points: (1) before occlusion of left anterior descending artery by balloon catheter (pre ischemia); (2) immediately after reperfusion with 2.5 μM/10 mL of δV1-1 (post ischemia); and (3) before sacrifice five days later (5 days post ischemia), using 6 Fr. of pig-tail catheter. LVG was recorded by 2 views, right anterior oblique and left anterior oblique. Ejection fraction (EF), the percent of blood ejected in a beat, during maximum contraction, of the total maximum present in the left ventricle, was analyzed by the software, Plus Plus (Sanders Data Systems), and the averages of two views were evaluated. Ejection fractions were calculated based on left ventricle dimensions and the results are shown in Fig. 10. Ejection fraction is a measure of how well the heart is functioning, with a higher ejection fraction indicative of a better functioning heart. An ejection fraction of less than 50% in a short period of time can suggest progression into a state of heart failure. Animals treated with δV1-1 (solid circles) exhibited a less pronounced decrease in ejection fraction than did the control animals treated with a scrambled peptide (open circles), suggesting that the peptide is effective to reduce or prevent damage to the cells and tissue due to ischemia. This is also evident from the data point at five days post ischemia, where animals treated with δV1-1 had an ejection fraction on par with that measured prior to ischemia and significantly higher than the untreated animals.

In summary, the *ex vivo* and *in vivo* studies show that δV1-1, when delivered before, during, or after ischemia, confers a substantial reduction of damage to the heart and brain induced by ischemia. Therefore, treatment with a δPKC peptide antagonist, such as δV1-1, δV1-2, δV1-5 peptides, provides a therapeutic treatment for tissues exposed to ischemia, such as occurs during cardiac ischemia.

D. *In vivo* Treatment for Inhibition of Stroke-Induce Damage

In another study performed in support of the invention, the ability of δV1-1 peptide (SEQ ID NO:4) to inhibit damage to the brain as a result of stroke was examined. In this study, described in Example 6, a rat cerebral ischemia model was used. Ischemia was induced using an intraluminal suture to occlude the ostium of the middle cerebral artery. δV1-1 conjugated to Tat peptide (SEQ ID NO:9) or the Tat peptide alone were injected into the carotid artery before and after a two hour occlusion period. The brain from each animal was harvested 24 hours later, stained, and examined. The results are shown in Figs. Figs. 11A-11B.

Fig. 11A is a digitized photograph of brains taken from untreated animals subjected to an induced stroke. The stained rat brain sections clearly demonstrated a middle cerebral artery territory infarct. The infarct area induced by the two hours of occlusion was reproducible between animals. Fig. 11B shows the brain sections from two animals treated with δV1-1 peptide prior to ischemia and at the end of the ischemic period. The significant reduction in infarct area is readily apparent.

Accordingly, the invention contemplates a method of reducing damage to tissue in the central nervous system, such as the brain, neurons, and glial cells, by administering a δPKC peptide antagonist, such as δV1-1, δV1-2, or δV1-5, prior to, during, or after a stroke. The peptide is effective to reduce the tissue damage, as evidenced by at least about a 10% reduction in infarct area, more preferably at least about a 25% reduction, and most preferably, at least about a 50% reduction in infarct area, when compared to untreated tissue exposed to the ischemic insult.

III. Method of Use

As described above, the peptides of the invention, δV1-1, δV1-2, δV1-5, and ψδRACK, act as translocation inhibitors or activators of δPKC. ψδRACK is an agonist, inducing translocation of δPKC to promote cell damage due to ischemia and/or hypoxia. δV1-1, δV1-2, and δV1-5 are antagonists, inhibiting δPKC translocation to prevent cell damage due to ischemia and resulting hypoxia.

It will be appreciated that the peptides can be used in native form or modified by conjugation to a carrier, such as those described above. Alternatively, one or two amino acids from the sequences can be substituted or deleted and exemplary modifications and derivatives and fragments for each peptide are given below.

For the ψδRACK peptide, identified as SEQ ID NO:6, potential modifications include the following changes shown in lower case: MkAAEDPM (SEQ ID NO:11), MRgAEDPM (SEQ ID NO:12), MRAgEDPM (SEQ ID NO:13), MRApEDPM (SEQ ID NO:14), MRAnEDPM (SEQ ID NO:15), MRAAdDPM (SEQ ID NO:16), MRAAEDPv (SEQ ID NO:17), MRAAEDPi (SEQ ID NO:18), MRAAEDPl (SEQ ID NO:19), and MRAAEDmp (SEQ ID NO:22), MeAAEDPM (SEQ ID NO:23), MdAAEDPM (SEQ ID NO:24), MRAAEePl (SEQ ID NO:25), MRAAEDPl (SEQ ID NO:26), MRAAEePi (SEQ ID NO:27), MRAAEePv (SEQ ID NO:28), MRAAEDPv (SEQ ID NO:29), and any combination of the above. The following modifications to ψδRACK are also contemplated

and are expected to convert the peptide from agonist to an antagonist: MRAAnDPM (SEQ ID NO:30), and MRAAqDPM (SEQ ID NO:31), MRAAEqPM (SEQ ID NO:32), MRAAEnPM (SEQ ID NO:33). Suitable fragments of ψδRACK are also contemplated, and SEQ ID NOS: 20, 21 are exemplary.

Accordingly, the term "a δPKC agonist" as used herein intends a ψδRACK peptide, which refers to SEQ ID NO:6 and to peptides having a sequence homologous to SEQ ID NO:6 and to peptides identified herein, but not limited to, as SEQ ID NO:11-19 and SEQ ID NO:21-29. The term a δPKC agonist further refers to fragments of these ψδRACK peptides, as exemplified by SEQ ID NOS:20-21.

For δV1-1, potential modifications include the following changes shown in lower case: tFNSYELGSL (SEQ ID NO:34), aFNSYELGSL (SEQ ID NO:35), SFNSYELGtL (SEQ ID NO:36), including any combination of these three substitutions, such as tFNSYELGtL (SEQ ID NO: 37). Other potential modifications include SyNSYELGSL (SEQ ID NO:38), SFNSfELGSL (SEQ ID NO:39), SNSYdLGSL (SEQ ID NO:40), SFNSYELpSL (SEQ ID NO:41). Other potential modifications include changes of one or two L to I or V, such as SFNSYEiGSv (SEQ ID NO:42), SFNSYEvGSi, (SEQ ID NO:43) SFNSYELGSv (SEQ ID NO:44), SFNSYELGSi (SEQ ID NO:45), SFNSYEiGSL (SEQ ID NO:46), SFNSYEvGSL (SEQ ID NO:47), aFNSYELGSL (SEQ ID NO:48), and any combination of the above-described modifications. Fragments and modification of fragments of δV1-1 are also contemplated, such as YELGSL (SEQ ID NO:49), YdLGSL (SEQ ID NO:50), fDLGSL (SEQ ID NO:51), YDiGSL (SEQ I, iGSL (SEQ ID NO:59)D NO:52), YDvGSL (SEQ ID NO:53), YDLpsL (SEQ ID NO:54), YDLglL (SEQ ID NO:55), YdLGSi (SEQ ID NO:56), YdLGSv (SEQ ID NO:57), LGSL (SEQ ID NO:58), iGSL (SEQ ID NO:59), vGSL (SEQ ID NO:60), LpSL (SEQ ID NO:61), LGIL (SEQ ID NO:62), LGSi (SEQ ID NO:63), LGSv (SEQ ID NO:64).

Accordingly, the term "a δV1-1 peptide" as used herein refers to a peptide identified by SEQ ID NO:4 and to peptides homologous to SEQ ID NO:4, including but not limited to the peptides set forth in SEQ ID NOS:34-48, as well as fragments of any of these peptides that retain activity, as exemplified by but not limited to SEQ ID NOS:49-64.

For δV1-2, potential modifications include the following changes shown in lower case: ALsTDRGKTLV (SEQ ID NO:65), ALTsDRGKTLV (SEQ ID NO:66), ALTTDRGKsLV (SEQ ID NO:67), and any combination of these three substitutions, ALTTDRpKTLV (SEQ ID NO:68), ALTTDRGrTLV (SEQ ID NO:69), ALTTDkGKTLV (SEQ ID NO:70), ALTTDkGkTLV (SEQ ID NO:71), changes of one or two L to I, or V

and changes of V to I, or L and any combination of the above. In particular, L and V can be changed to V, L, I R and D, E can change to N or Q.

Accordingly, the term "a δV1-2 peptide" as used herein refers to a peptide identified by SEQ ID NO:5 and to peptides homologous to SEQ ID NO:5, including but not limited to the peptides set forth in SEQ ID NOS:65-71, as well as fragments of any of these peptides that retain activity.

For δV1-5 (SEQ ID NO: 7), potential modifications include those similar to the modifications described for δV1-2. The term "a δV1-5 peptide" as used herein refers to SEQ ID NO:7 and to peptides homologous to SEQ ID NO:7 as well as fragments thereof that retain activity.

Accordingly, the term "a δPKC antagonist" as used herein intends a δPKC peptide, which refers to any a δV1-1 peptide, a δV1-2 peptide and a δV1-5 peptide.

In still other embodiments, the peptide can be part of a fusion protein or a transport protein conjugate. Typically, to form a fusion protein, the peptide is bound to another peptide by a bond other than a Cys-Cys bond. An amide bond from the C-terminal of one peptide to the N-terminal of the other is exemplary of a bond in a fusion protein. The second peptide to which the δPKC agonist/antagonist peptide is bound can be virtually any peptide selected for therapeutic purposes or for transport purposes. For example, it maybe desirable to link the δV1-1 peptide to a cytokine or other peptide that elicites a biological response.

Where the peptide is part of a conjugate, the peptide is typically conjugated to a carrier peptide, such as Tat-derived transport polypeptide (Vives *et al.,* 1997), polyarginine (Mitchell *et al.*, 2000; Rolhbard *et al.,* 2000) or Antennapedia peptide by a Cys-Cys bond. See U.S. Patent No. 5,804,604. In another general embodiment, the peptides can be introduced to a cell, tissue or whole organ using a carrier or encapsulant, such as a liposome in liposome-mediated delivery.

The peptide may be (i) chemically synthesized or (ii) recombinantly produced in a host cell using, *e.g.*, an expression vector containing a polynucleotide fragment encoding said peptide, where the polynucleotide fragment is operably linked to a promoter capable of expressing mRNA from the fragment in the host cell.

In another aspect, the invention includes a method of reducing ischemic injury to a cell, tissue or whole organ exposed to hypoxic conditions. The method includes introducing into the cell, tissue or whole organ prior to exposure to hypoxic conditions, a

therapeutically-effective amount of an isozyme-specific δPKC antagonist, such as δV1-1, δV1-2, δV1-5, or any of the modification, derivatives, and fragments of these peptides described above. The δPKC antagonist inhibits δPKC, resulting in protection of the cell, tissue or whole organ by reducing ischemic injury to the cell. The reduction of ischemic injury is measured relative to the ischemic injury suffered by a corresponding cell, tissue or whole organ that did not undergo δPKC antagonist peptide pretreatment.

It will be appreciated that the dose of peptide administered will vary depending on the condition of the subject, the timing of administration (that is, whether the peptide is administered prior to, during, or after an ischemic event). Those of skill in the art are able to determine appropriate dosages, using, for example, the dosages used in the whole organ and animal studies described herein.

The method can be practiced with a variety of cell types, including cardiac cells, central nervous system (CNS) cells (e.g., neurons, glial cells), kidney cells and the like. A variety of tissue or whole organs can be treated, including but not limited to the brain, heart, eye, and kidney.

The peptides can be administered to the cell, tissue or whole organ *in vitro, in vivo,* or *ex vivo*. All modes of administration are contemplated, including intraveneous, parenteral, subcutaneous, inhalation, intranasal, sublingual, mucosal, and transdermal. A preferred mode of administration is by infusion or reperfusion through arteries to a target organ, such as through the coronary arteries to an intact heart.

In yet another aspect, the invention includes a method of enhancing ischemic injury to a cell, tissue or whole organ exposed to hypoxic conditions. This method is relevant to, for example, the treatment of solid tumors in subjects. The method also finds use in *in vitro* or *in vivo* research where damage to a cell or tissue is desired. The method includes introducing into the cell, tissue or whole organ a therapeutically-effective amount of an isozyme-specific δPKC agonist, such as ψδRACK (SEQ ID NO:6)or any of the peptides obtained from a modification to ψδRACK as discussed above. The extent of enhanced ischemic injury is measured relative to the ischemic injury suffered by a corresponding cell, tissue or whole organ untreated with a δPKC agonist.

IV. <u>Identification and Screening of Test Compounds</u>

In another aspect, the invention includes methods of identifying compounds effective to induce protection of a cell or tissue from hypoxic/ischemic damage or to enhance hypoxic or ischemic damage in a cell or tissue.

In the first method, the δPKC-specific agonists δV1-1, δV1-2, δV1-5 or any of the modifications of these peptides described above, are used to identify compounds effective to inhibit δPKC translocation in cells and/or to competitively displace the peptide from Annexin V (SEQ ID NO:72) or other δRACK and/or to prevent or inhibit the peptide from binding to such a δRACK. Such compounds find use as therapeutic agents to inhibit δPKC translocation and/or function to thereby induce protection of cells or tissues from damage due to ischemia. The compounds also find use as screening tools to identify other peptides or compounds suitable for the same purpose.

In this method, a δPKC peptide containing a δRACK binding site, such as Annexin V, is brought into contact with a δPKC antagonist peptide with the δRACK binding site, such as δV1-1, δV1-2, or δV1-5, in the presence and absence of a test compound. The interaction of the test compound with the peptide having the δRACK binding site is monitored and/or the catalytic activity of the δPKC agonist or the test compound is monitored. Generally, the test compound is identified as being effective to induce protection from an ischemic or an hypoxic event if, in the presence of the test compound, binding of the peptide antagonist to the δRACK binding site is decreased, relative to binding in the absence of the test compound. Alternatively, the catalytic activity of the components can be monitored. For example, the phosphorylation activity of the peptides can be monitored. If the ability of the test compound to increase phosphorylation, or some other catalytic activity subsequent to binding, is increased relative to activity in the absence of the test compound then the compound is identified as being effective to induce protection from damage caused by either a hypoxic or an ischemic event.

In another method, the agonist peptide ψδRACK can be used to identify compounds effective to enhance hypoxic or ischemic damage in a cell or tissue. In this method, a ψδRACK agonist peptide is brought into contact with a δPKC peptide containing a δRACK binding site in the presence and absence of a test compound. The test compound, if able to decrease binding of the peptide agonist to the δRACK binding site relative to binding in the absence of the test compound, is identified as being effective to enhance damage due to ischemia. Suitable ψδRACK peptides include the peptide identified as SEQ ID NO:6, fragments, and derivatives thereof, including but not limited to those set forth in SEQ ID NOS:10-24.

ψδRACK-like compounds can also be identified by measuring its effect on the catalytic activity of δPKC *in vitro*. The desired compound will increase the catalytic

activity of δPKC in the presence of limiting amounts of δPKC co-factors (Ron *et al.*, 1995). Catalytic activity refers to the ability of the peptide to phosphorylate another protein or substrate.

Experimental details of a similar screening method are set forth in U.S. Patent No. 6,165,977, and this portion on Col. 14, line 45-Col 15, line 54 is incorporated by reference herein. In brief, and by way of example for identifying a compound effective to protect a cell or tissue from ischemia, δPKC is immobilized inside the wells of a multiwell plate by introducing a solution containing δPKC into the plate and allowing the δPKC to bind to the plastic. The wells may be precoated with substances that enhance attachment of δPKC and/or that decrease the level of non-specific binding.

The plate is then incubated with a blocking solution (containing, for example bovine serum albumin) and then washed several times. A solution containing reporter-labelled (*e.g.*, radiolabelled of fluorescently-tagged) peptide δV1-1 (SEQ ID NO: 4) and, in the test wells, as opposed to the control wells, a test compound is added. Different wells may contain different test compounds or different concentrations of the same test compound. Each test compound at each concentration is typically run in duplicate and each assay is typically run with negative (wells with no test compound) as well as positive (wells where the "test compound" is unlabeled peptide) controls. The free peptide is then washed out, and the degree of binding in the wells is assessed.

A test compound is identified as active it if decreases the binding of the peptide, *i.e.*, if its effect on the extend of binding is above a threshold level. More specifically, if the decrease in binding is a several-fold different between the control and experimental samples, the compound would be considered as having binding activity. Typically, a 2-fold or 4-fold threshold difference in binding between the test and control samples is sought.

Detection methods useful in such assays include antibody-based methods, direct detection of a reporter moiety incorporated into the peptide, such as a fluorescent label, and the like.

A variety of test compounds may be screened, including other peptides, macromolecules, small molecules, chemical and/or biological mixtures, fungal extracts, bacterial extracts or algal extracts. The compounds can be biological or synthetic in origin.

From the foregoing, it can be seen how various objects and features of the invention are met. New activator and inhibitor peptides of δPKC translocation and function were identified. The peptides can be delivered *in vivo* or *ex vivo* to achieve a functional

inhibition or activation of δPKC. For example, delivery of the peptides to an intact heart via the coronary artery permits the peptides to act as a direct peptide modulator of protein-protein interactions intracellulary. It was also found that inhibition of δPKC by delivery of a δPKC antagonist reduces tissue damage due to an ischemic event. It is noteworthy that δPKC and εPKC (previously described in the art) exhibit an opposing effect in response to ischemia, yet activation of both isozymes leads to a similar form of cardiac hypertrophy. This was particularly unexpected, because both isozymes are activated by ischemia as well as by stimuli that lead to cardioprotection from ischemia (Gray, M.O. *et al.*, Chen, C.-H. *et al*). δPKC and εPKC are opposing forces and a balance between these opposing forces likely determines the outcome to the ischemic insult, where protection occurs when activation of εPKC exceeds that of δPKC. During a long ischemic period, there may be an advantage to induce cell death, which will result from a time-dependent increase in the activity of δPKC relative to that of εPKC. In that way, the limited amounts of oxygen, glucose and other nutrients could be used by the remaining, less damaged, cells, ultimately leading to an improved outcome to the organ.

V. Examples

The following examples further illustrate the invention described herein and are in no way intended to limit the scope of the invention.

Example 1

Activity of δV1-1, δV1-2 and ψδRACK

A. Peptide Preparation

δV1-1 (SEQ ID NO:4) and ψδRACK (SEQ ID NO:6) were synthesized and purified (>95%) at the Stanford Protein and Nucleic Acid Facility. The peptides were modified with a carrier peptide by cross-linking via an N-terminal Cys-Cys bond to the *Drosophila* Antennapedia homeodomain (SEQ ID NO:8; Théodore, L., *et al.*; Johnson, J. A. *et al.*, 1996b). In some studies not reported here, the peptides were lined to Tat-derived peptide (SEQ ID NO:9).

B. Peptide Delivery Into Cells

Primary cardiac myocyte cell cultures (90-95% pure) were prepared from newborn rats (Gray, M.O. *et al.*; Disatnik M.-H. *et al.*). The peptides δV1-1 and ψδRACK were introduced into cells at an applied concentration of 500 nM in the presence and absence of

phorbol 12-myristate 13-acetate (PMA) at concentrations of 3 nm and 10 nm, respectively, for 10-20 minutes. In a third set of cells, the peptide δV1-1 was applied at a concentration of 500 nM in the presence and absence of 500 nM ψδRACK.

Translocation of δPKC isozyme was assessed by using δPKC isozyme-specific antibodies in Western blot analysis (Santa Cruz Biotechnology). Western blot analysis of cystosolic and particulate fractions of treated cells was carried out as described by Johnson *et al.*, 1995. Subcellular localization of dPKC isozymes was assessed by chemiluminescence of blots probed with anti-δPKC, anti-αPKC and anti-εPKC antibodies. Amounts of PKC isozymes in each fraction was quantitated using a scanner and translocation is expressed as the amount of isozymes in the particulate fraction over the amount of isozymes in non-treated cells. Changes in translocation of δPKC isozyme were also determined by immunofluoresence study of treated and fixed cells (Gray *et al.*, 1997). Translocation was determined by counting over 100 cells/treatment in a blinded fashion. The results are shown in Fig. 2A-2B, Figs. 3A-3B and Figs 4A-4B.

Example 2

Peptide Administration to Isolated Cardiac Myocytes

The peptides δV1-1 and ψδRACK were prepared as described in Example 1.

Adult male Wistar rat cardiomyocytes were prepared on a Langendorff apparatus (van der Heide, R.S. *et al.*) by collagenase treatment (Armstrong, S. *et al.*). The cells were treated with δV1-1 at concentrations of 10 nM, 100 nM, 500 nM, and 1 μM in the presence or absence of 1 μM ψδRACK. βPKC-selective activator was used as a control.

For stimulated ischemia, adult myocytes treated in microcentrifuge tubes with δV1-1 and/or ψδRACK peptides conjugated to the carrier were washed twice with degassed glucose-free incubation buffer and pelleted. On top of a minimal amount of buffer, the cell pellets were overlaid with either a micro-balloon (Sig Manufacturing, Montezuma, IA) or with degassed buffer satured with nitrogen, and sealed with an airtight top. Tubes were then incubated at 37C for either 180 minutes or 90 minutes.

Cell damage was assessed by an osmotic fragility test by measuring the uptake of trypan blue added in a hypotonic (85 mosM) solution. The results are shown in Figs. 5A-5B. Similar results were also obtained by using a live-dead kit (Molecular Probes) or measuring the release of lactose dehydrogenase to the medium using a kit (Sigma) as previously described (Chen, *et al.,* 1999; Gray *et al.*, 1997; Mackay *et al.*, 1999).

Example 3

Ex vivo Peptide Administration to Whole Hearts and Effect on Cell Damage

Adult, male rats were anesthetized with i.p. avertin, and their hearts were rapidly removed and cannulated via the aorta for perfusion as described in the art (Colbert, M. C. *et al.*) using Langendorff set-up. Care was taken to have the hearts perfused within 90 seconds of removal. The hearts were perfused with oxygenated Krebs-Henseleit solution comprised of, in nmol/L, NaCl 120; KCl 5.8; $NaHCO_3$ 25; NaH_2O_4 1.2; $MgSO_4$ 1.2; $CaCl_2$ 1.0; and dextrose 10, pH 7.4 at 37 C.

After a 10-20 minute equilibration period, the hearts were perfused with δV1-1 peptide (SEQ ID NO:4) or with ψδRACK peptide (SEQ ID NO:6), prepared as described in Example 1 but conjugated to a Tat-derived peptide (Tat 47-57, SEQ ID NO:9), for 20 minutes. Perfusion was maintained at a constant flow of 10 mL/min with Krebs-Hanseleit solution containing 0.5 μM of the appropriate peptide. The Langendorff method employed used retrograde flow from the ventricle to the aorta and into the coronary arteries, bypassing the pulmonary arteries.

To induce global ischemia, flow was interrupted for 30 minutes. After the ischemic event, the hearts were re-perfused for 30-60 minutes. During reperfusion, ischemia-induced cell damage was determined by measuring the activity of creatine phosphokinase (CPK) (absorbance at 520 nm) in the perfusate using a Sigma kit. As controls, some *ex vivo* hearts were left untreated, or maintained under normoxia conditions, or treated with the Tat-carrier peptide alone, or treated with Tat-carrier peptide conjugated to a scrambled δV1-1 peptide. The results are shown in Figs. 6A-6B.

Example 4

Ex vivo Peptide Administration to Whole Hearts and Effect on Functional Recovery

Rat hearts were isolated as described in Example 3. The left ventricular pressure and its real-time derivative (*dP/dt*) were monitored via a latex balloon placed in the ventricular cavity and at a constant heart rate by pacing (3.3 Hz) and at a constant coronary flow (10 ml/min.). The hearts were subjected to 20 minutes of ischemia and 30 minutes of re-perfusion. During the first 20 minutes of reperfusion, 500 nM of δV1-1 or vehicle control was delivered. The results are shown in Figs. 7A-7B.

Example 5

In vivo Administration of δV1-1 After Ischemia

Adult female pigs, 35-40kg in weight, were anesthetized and a catheter was introduced through the carotid artery into the heart. Using conventional intervention cardiology techniques, a wire was placed through a catheter and into the left anterior descending artery. A balloon was run over this wire to a site of occlusion where it was then inflated to block blood flow for 30 minutes. During the last 10 minutes of the 30-minute occlusion, either a control comprised of the carrier peptide alone or δV1-1 peptide (conjugated to a carrier Tat peptide as described in Example 3 was delivered by slow diffusion (1mL/min) directly downstream of the occlusion. Approximately 20μg of δV1-1 peptide (~400ng per kg body weight) was administered.

After 30 minutes of occlusion, the balloon was removed and pigs were left to recover from surgery. Five days later, the pigs were euthanized and hearts were harvested. After heart removal, the LAD was occluded. With the occlusion in place, Evans Blue dye, which stains all areas not at risk of infarct in blue while leaving all areas with no access to blood flow red, was infused. Hearts were then cut into slices and stained with a tetrazolium red dye which stains all live areas red and infarcted dead tissue in white. Each heart had multiple tissue slices with distinctive areas marking the area at risk for ischemia and the infarcted area. From this the percent infarct per area at risk for each slice and for the entire heart was determined. The results are shown in Figs. 9A-9C.

Example 6

In vivo Administration of δV1-1 to Rats for Stroke Damage Protection

A. Cerebral Ischemia Model

Adult male Sprague-Dawley rats weighing between 280-320 g were used. Animals were maintained under isofluorane anesthesia during all surgical procedures. Physiological parameters were monitored and maintained in the normal range. Rectal temperature was also measured. At the completion of the experiment, the animals were euthanized with a barbiturate overdose and prepared for histological analysis.

B. Focal model

Ischemia was induced using an occluding intraluminal suture. An uncoated 30 mm long segment of 3-0 nylon monofilament suture with the tip rounded by flame was inserted into the stump of the common carotid artery and advanced into the internal carotid artery approximately 19-20 mm from the bifurcation in order to occlude the ostium of the middle

cerebral artery. Sham control animals underwent similar anesthesia and surgical manipulation, but did not experience ischemia. At the end of a 2 hour ischemic period, the suture was removed and the animal allowed to recover. Brains were harvested after 24 hrs of reperfusion.

C. Peptide delivery

δV1-1 (SEQ ID NO:4) conjugated to Tat peptide (0.05 mL, SEQ ID NO:8) or Tat carrier control peptide (50μL of 10μM solution in saline) were injected into the carotid artery either immediately before or before and after the 2 hours occlusion. The final blood concentration of δV1-1 was 1 μM.

D. Histology

Animals were perfused with heparinized saline and brains removed and sectioned into 2 mm thick slices. To assess ischemic injury, brain sections were stained with cresyl violet or with triphenyl tetrazolium chloride, a live tissue stain to indicate the regions of infarct. Areas of infarction (white) were then measured using an image analysis system previously described (Yenari, M.A. *et al.,* 1998; Maier, C. *et al.,* 1998). The results are shown in Figs. 11A-11B.

Although the invention has been described with respect to particular embodiments, it will be apparent to those skilled in the art that various changes and modifications can be made without departing from the invention.